CARACTERIZATION OF CULTIVATED STRAINS OF A NEW EDIBLE MUSHROOM: *STROPHARIA RUGOSO-ANNULATA*. I. PROTEIN VARIABILITY.

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ABSTRACT Fourteen cultivated strains of the edible mushroom *Stropharia rugoso-annulata* have been collected from different research groups in Europe and compared to evaluate intraspecific variability. Electrophoretic analysis of soluble mycelium proteins was found to be an efficient tool to describe the variability. The relative similarities between total protein patterns and esterase isozymes, as calculated from the Rogger's and Jaccard's coefficients, and the resultant dendrograms allowed us to characterize two main clusters of strains: the first one consisted of seven strains from Belgium, Germany, France and Hungary and the second included four strains from Germany and Hungary. The three remaining strains isolated following total protein analysis could be considered with respect to esterase pattern as close to one or the other group. The esterase enzyme study appeared as a more powerful tool to assess the intraspecific variability. This is of special interest for further work aimed at improving the commercial quality of *Stropharia*.

KEY WORDS basidiomycetes, biodiversity, edible mushroom, esterases, protein electrophoresis, *Stropharia rugoso-annulata*

RÉSUMÉ La diversité au sein de l'espèce cultivée *Stropharia rugoso-annulata* a été évaluée à partir de 14 souches d'intérêt commercial provenant de différents laboratoires de recherche européens. Les profils électrophorétiques des protéines solubles (protéines totales et système estérasique) sur gel de polyacrylamide ont permis de décrire la variabilité protéique de l'espèce. Sur la base des protéines totales, les degrés de similarité entre souches, calculés selon les coefficients de Roggers et Jaccard, ont conduit à l'obtention de deux groupes principaux. Le premier groupe est formé de 7 souches d'origines belge, allemande, française et hongroise et le second, de 4 souches provenant d'Allemagne et de Hongrie. Le profil enzymatique des trois souches non classées permet de supposer qu'elles sont proches tantôt du premier groupe, tantôt du second. Le potentiel de ces souches devra maintenant être évalué en culture, il pourra ensuite être exploité lors de travaux d'amélioration commerciale de l'espèce.

MOTS CLÉS basidiomycètes, biodiversité, champignon cultivé, électrophorèse des protéines, estérases, *Stropharia rugoso-annulata*

INTRODUCTION

Stropharia rugoso-annulata Farlow ex Murrill is a homobasidiomycete, in the order Agaricales and the family *Strophariaceae*. The domestication of *Stropharia rugoso-annulata* for mushroom production was first proposed by Puschel (1969) at Dieskau (Germany). Original strains were collected from the natural environment of the saprophytic basidiomycete. Subsequently, a number of works focused on cultural techniques in the 70's, mostly in Czechoslovakia (Staneck,1974), Hungary (Balazs, 1974) and Poland (Szudyga, 1978).

In Europe, the white button mushroom dominates commercial production. However, worldwide interest in mushroom cultivation is increasing and the diversification of European production has become necessary. Among higher fungi, *Pleurotus sp.* (Oyster) and *Lentinus edodes* (Shiitake) are known as economically important genera and are thoroughly studied in many laboratories. The edible mushroom, *Stropharia rugoso-annulata*, could also contribute to the diversification of cultivated mushrooms through the development of specific cultivation techniques (Poppe & Sedeyn, 1987, Bonenfant, 1993). Until now, there has been no information on the biological diversity within collections of *Stropharia* strains. The purpose of our investigations was to evaluate the natural variability in this cultived species with regard to advances in selection, improvement of strains, and, the necessity for efficient protection of commercial varieties. When only the vegetative stage is available, a statistical study of electrophoretic profiles of soluble proteins or isozyme activities is generally seen as a valuable tool in taxonomy (Royse & May, 1987, Itavaara 1988, May & Royse, 1988, Itavaara, 1990). In the present paper, fourteen commonly used strains of *Stropharia rugoso annulata* from various origins are characterized by electrophoretic protein patterns. We first describe the total protein pattern of each strain with the aim of assessing the degree of similarity between them. Then, the results obtained through total protein analysis are compared to those of esterase zymograms, this enzyme system being known to exhibit a large number of isozymes.

MATERIALS AND METHODS

Biological material. Fourteen dikaryotic strains of *Stropharia rugoso-annulata* were collected from different European research laboratories and from spawn growers. The strains under study were chosen because of their use in cultivation and their commercial interest. However, no indications about varietal or genetic diversity between these strains are available. The strains and their origins are listed in Table I. The abbreviations are those known in the laboratories from which the strains are supplied.

Culture media and growth conditions. Two types of media were used for vegetative multiplication: a liquid medium (10 g/l malt extract, M1) and a solid one (20 g/l malt extract added with 15 g/l agar, M2). The agar medium (M2) was autoclaved for 20 min at 120 °C before being poured in sterile petri dishes (about 20 ml/dish). For the liquid medium (M1), 25 ml aliquots were transferred to 65 ml. flasks. Then the flasks were stoppered with cotton plugs and autoclaved at 120° C for 20 min. A piece of mycelium (5 mm in diameter) grown on the sterile solid medium (M2) was transferred aseptically to the flasks containing liquid medium (M1), and the culture was placed for 21 days at 20 °C in the dark.

Sample preparation. The mycelia were harvested, filtered and washed with distilled water. The samples were then freeze-dried and stored at -20°C for subsequent analysis.

Abbreviations	Origin
IT	Lab. voor Fytopathologie, University of Gent (Belgium)
606 ; 610	"Mycoblank", Merlebeke (Belgium)
L3G	Versuchsanstalt fur Pilzanbau, Krefeld (Germany)
Z10	Institut für bodenbiologie, Braunschweig (Germany)
WD , GD , GRD , T54	VEG - Champignonzucht, Dieskau (Germany)
GH , WH , LH	Research Center for Agrobotany, Kecskemet (Hungary)
F700	"Royal Champignon", Saumur (France)
LM	C.R. Champignons, I N.R.A., Bordeaux (France)

Table 1 Origin of the commercial strains of *Stropharia rugoso annulata* collected for the study

Twenty milligrams of freeze-dried mycelium were weighed out and ground with liquid nitrogen. Mycelium powder was then homogenized at 2°C in 1.0 ml of extraction buffer. After centrifugation at 17600 g, the pellet was discarded and the supernatant was analysed as the total protein extract. With the aim of optimizing protein extraction, four different extraction buffers were tested: two phosphate buffers (50 mM) with pH adjusted to either 6.0 or 7.5, and two Tris (Tris hydroxymethyl aminomethane) buffers (Tris-HCl, 50 mM, pH 7.5 and Tris 25 mM-Glycine 192 mM, pH 8.3).

Protein determination. Protein content was determined as described by Bradford (Bradford, 1976) using Bovine Serum Albumine (BSA) as the standard.

Polyacrylamide gel electrophoresis. Protein samples containing 50 mg of soluble proteins were subjected to non-denaturing electrophoresis on a 1 mm thick, 7.5 % polyacrylamide gel overlaid with a 4.5 % polyacrylamide stacking gel using the discontinuous buffer system of Laemmli (Laemmli, 1970). Electrophoresis was carried out at 5°C with a constant 15 mA current per slab.

Protein and esterase staining. After electrophoresis, the gel slabs were placed directly into solutions for the staining of specific proteins. Coomassie Brilliant Blue R 250 (Sigma) was used for staining total proteins. To detect esterase activities, the gel slab was incubated for 1 hour at 37°C in a staining mixture containing: 0.1M phosphate buffer, pH 6, 1mg/ml Fast Blue RR salt, 1 % a naphthyl acetate and 1% b-naphthyl acetate. The naphthol radical of the carboxylic esters was released by esterases and combined with diazonium salt (Fast Blue RR) to form an insoluble product. The resulting bands on the gel slab were dark brown or pink depending on whether they involved a or b-naphthyl acetate, respectively.

Numerical analysis. Relative electrophoretic mobilities (Rm) of the protein bands were calculated and the absence or presence of each band was recorded. Jaccard and Rogger's similarity coefficients for all possible pairs of isolates were calculated according to Bidault

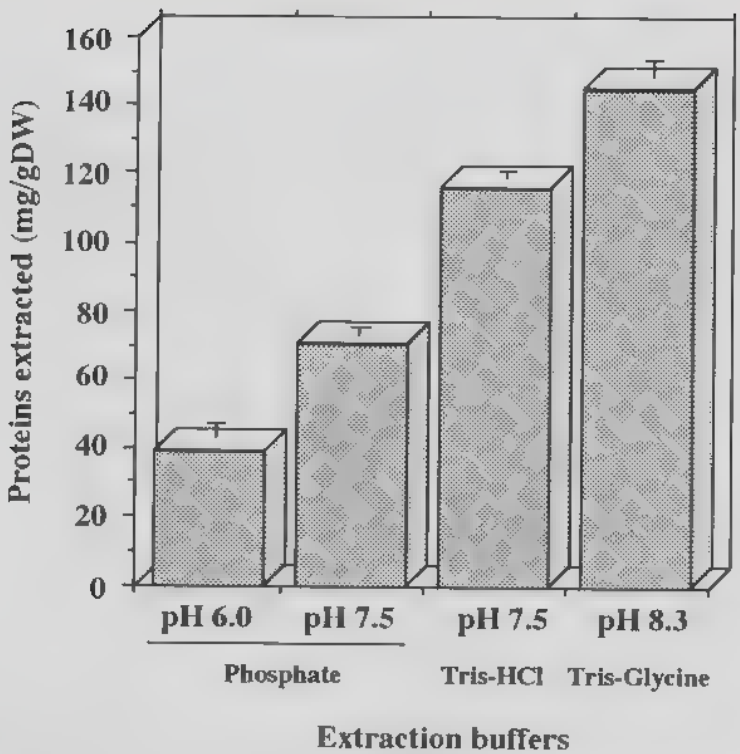

Fig 1 Effect of buffer composition on the quantity of mycelium protein extracted Data are means ± standard deviation of three replicates

(Bidault, 1971). The degree of similarity between strains, as based on Jaccard's coefficient, was used to generate a dendrogram with a STAT ITCF computer program according to the 'weighted mean distance' method.

RESULTS

Mycelial protein extraction. When applied to the same strain, the four extracting buffers tested showed different patterns of extractibility (Fig 1) Tris buffers seemed to be more efficient for mycelial protein extraction, as compared to phosphate buffers with similar pH value Considering the effect of buffer pH on protein extraction, it was found that alkaline buffers are more powerful than neutral or weakly acidic ones For these reasons, the comparative study of both strains on the basis of protein content and pattern has been performed with Tris glycine extracts (pH 8 3) This extraction buffer was also used as the running buffer for electrophoretic studies It has to be noted that the addition of polyvinyl polypyrrolydone (PVPP) to the extraction buffer did not improve the quality of electrophoretic pattern of mycelial proteins In addition, the use of SDS (sodium dodecyl sulfate)

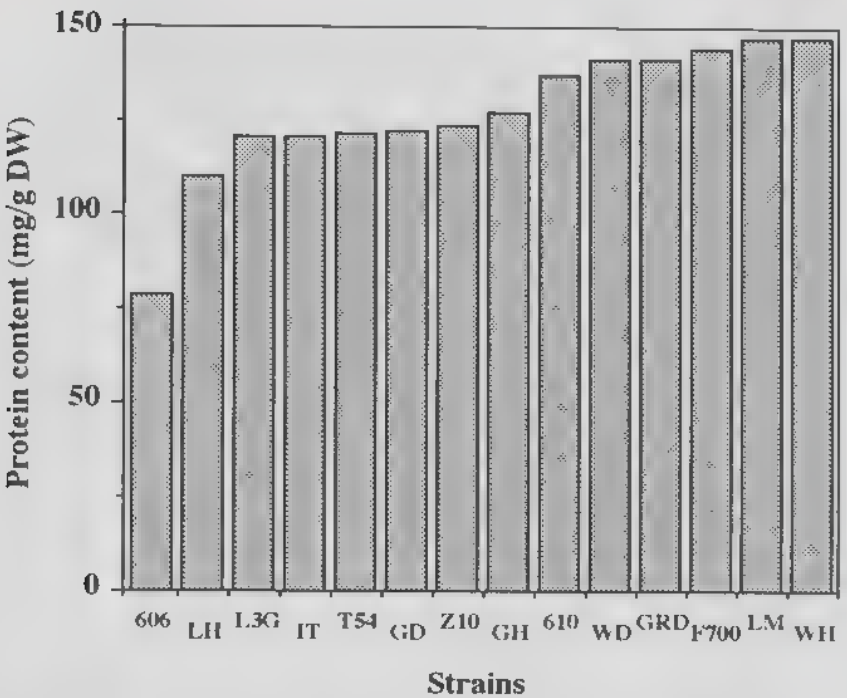

Fig. 2. Protein content (mg/g dry weight) of mycelium of *Stropharia rugoso-annulata* strains after 21 days of cultivation on MI medium

or MF (b mercaptoethanol) increased considerably the number of bands on protein pattern. This would allow to compare strains only on the basis of band intensity rather than the commonly used presence/absence criteria. Therefore, soluble proteins of *Stropharia* strains have been studied under non-denaturing conditions.

Protein content of the mycelium. The dry weight of mycelia grown for 21 days in the liquid MI medium ranged from 40 to 60 mg per flask, depending on the strains. The content of soluble proteins for each strain is represented in Figure 2. This trait revealed a large heterogeneity between strains, with 606 exhibiting the lower protein content (7.7 DW) and a group of strains (610, WD, GRD, F700, LM, WH) being rich in soluble proteins (> 14% DW).

Total protein patterns. Coomassie Brilliant Blue reagent used for total protein staining, allowed the discrimination of eleven different profiles from the fourteen strains under study (Fig. 3). Three bands (not represented on Figure 3) were found in all patterns and the analysis of 15 other protein bands allowed us to assess intraspecific variation. Some similarities were observed among patterns, from which the succession of three minor bands (Rm 0.46, 0.48, 0.50) and two other ones (Rm 0.87, 0.89) could help to characterize four strains (LH, GH, L3G, GD). On the other hand, the Belgian strain 606 was clearly distinguishable from the others, in that it exhibited only a few number of protein bands.

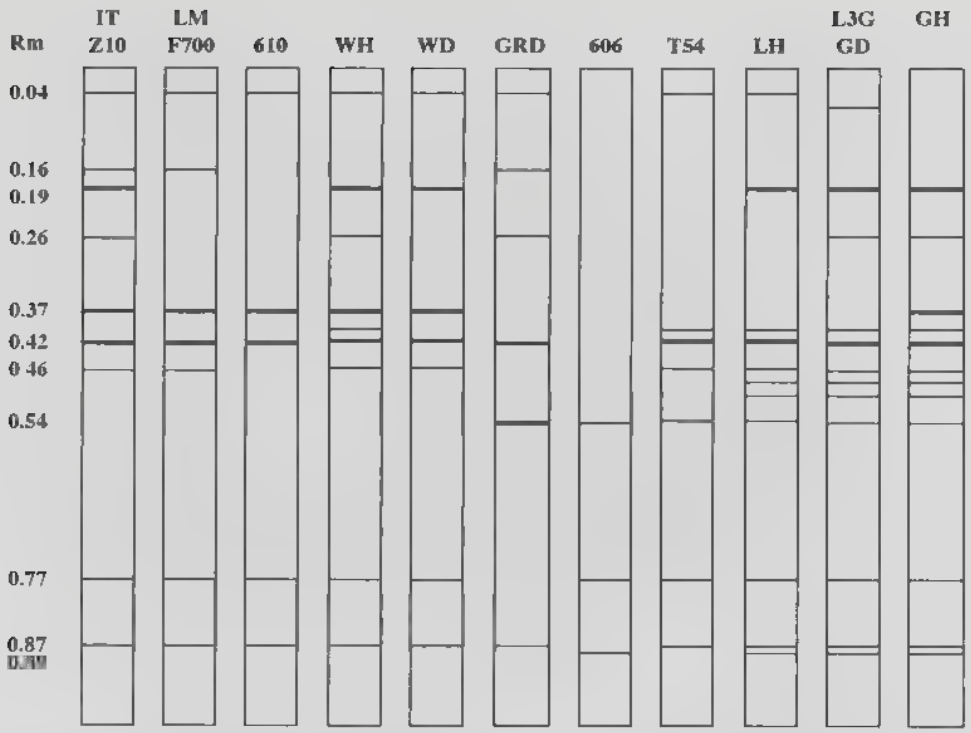

Fig 3 Diagram of electrophoretic patterns of total proteins from 14 *Stropharia rugoso-annulata* strains : only the bands showing variations are represented

The relative similarities between protein patterns of these strains were determined by the calculation of Rogger and Jaccard's coefficients (Fig 4) The latter always gave higher values than the former However, similar trends were found in that two groups of strains having high similarity indexes could be separated The resultant dendrogram based on cluster analysis of these phenotypic similarities is represented in Figure 5

In terms of phenotypic characterization, two main clusters of strains were identified by the analysis of banding pattern for heterozygous individuals The first cluster included seven strains two Belgian strains (IT, 610), two German strains (Z10, WD), two French strains (LM, F700), and one Hungarian strain (WH) The second major cluster includes four strains, two German (GD, L3G) and two Hungarian (LH, GH) Two other German strains (T54 and GRD) have been classified in an intermediary position between these two main clusters, and a single strain from Belgium (606) appeared to be more divergent according to this method

Esterase patterns. The electrophoretic variation of esterase isoenzymes between cultivated strains of *Stropharia rugoso-annulata* was studied Depending on the strains, a maximum of 15 bands with enzyme activity appeared on the gels (Fig 6) Among these bands, five corresponded to the b-esterase activity The isozyme with the highest molecular

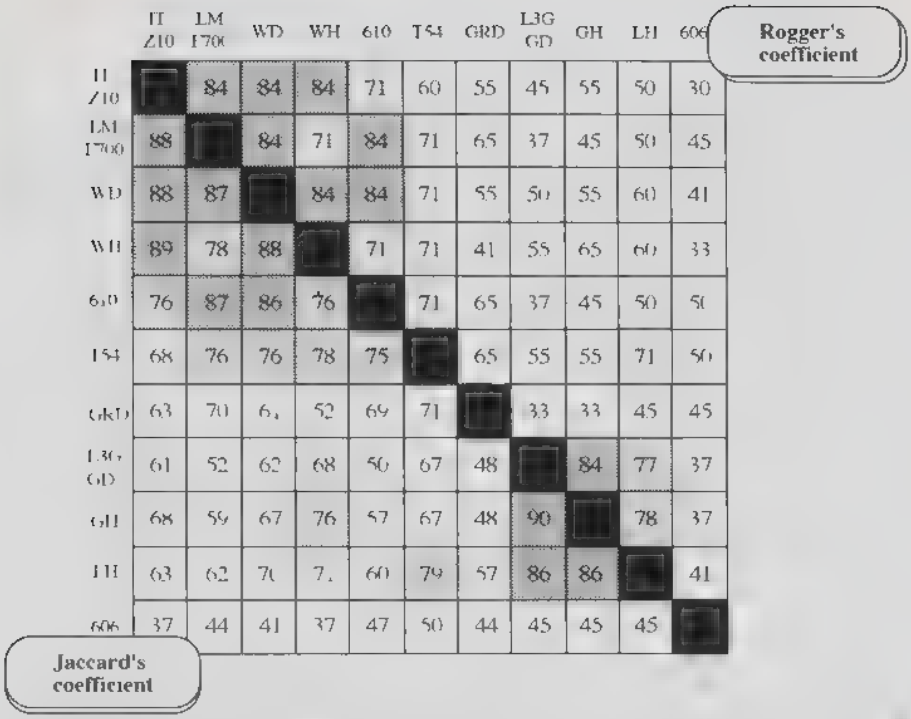

Fig. 4. Comparison between Rogger and Jaccard's coefficients calculated from total protein > 80 %, 75 to 80 %, 70 to 75 %

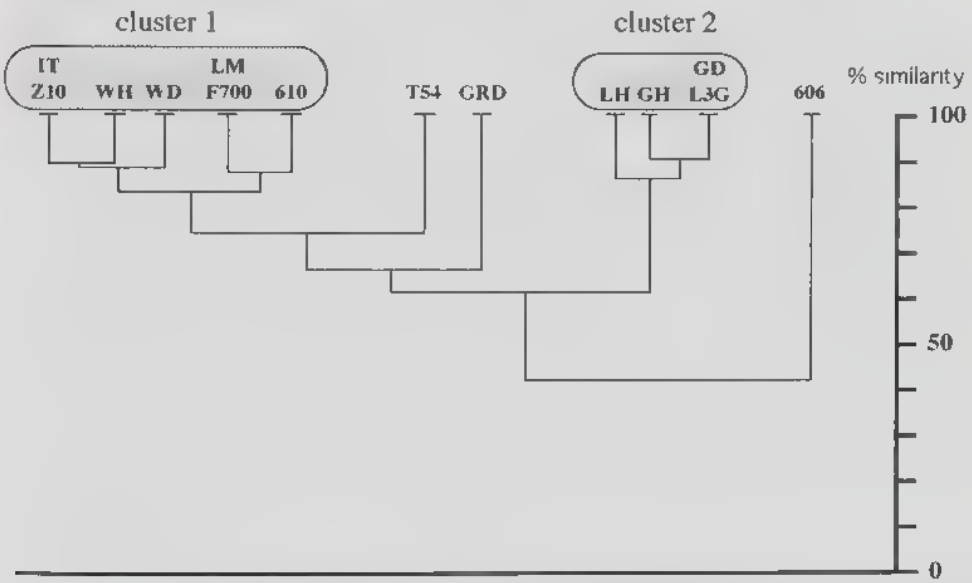

Fig. 5. Dendrogram of Jaccard's similarities for 14 *Stropharia rugoso-annulata* strains based on total protein patterns.

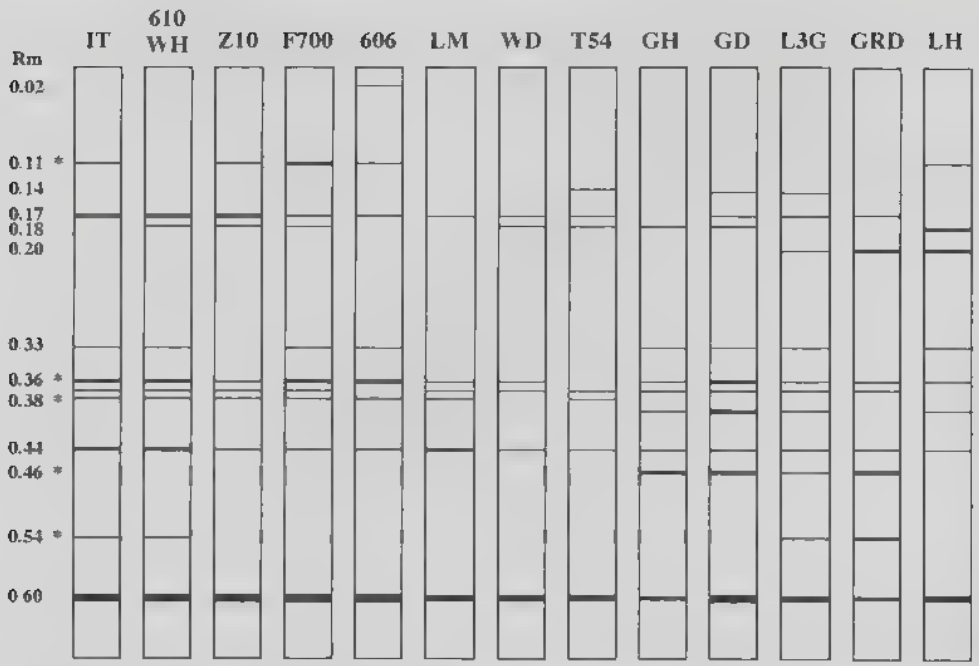

Fig 6 Diagram of electrophoretic patterns of esterase isozymes from 14 *Stropharia rugoso-annulata* strains ; *, pink band indicating β-esterase isozyme

weight was recorded in only one strain, 606 In contrast, the lightest isozyme (as well as that with a Rm of 0 44) was present in every strain It is also the most abundant form of esterase, as shown by the intensity of the corresponding band. Unlike what was seen with protein patterns, a large diversity of band width and intensity was found Moreover, strains belonging to the second cluster previously defined could be characterized by the presence of one esterase isoform, which showed a Rm of 0 46 A relatively low variation could be detected between the strains included in the first cluster as determined by total protein patterns (Fig 7) Interestingly, the enzyme pattern of Belgian strain 606 (and to a lesser extent that of T54) was not very different from that of these strains. Thus, the 606 strain could be considered, on the basis of esterase pattern, as close to the first cluster of strains.

In contrast to the strains from the first group, the remaining strains are more distinguishable one from another, on the basis of the esterase zymograms.

DISCUSSION

Taxonomic identification of the species *Stropharia rugoso-annulata* was primarily based on the morphological study of the reproductive structure (Puschel, 1969, Gumberteau, 1978, Kuhner & Romagnesi, 1978; Lamoure, 1984) However, morpholo-

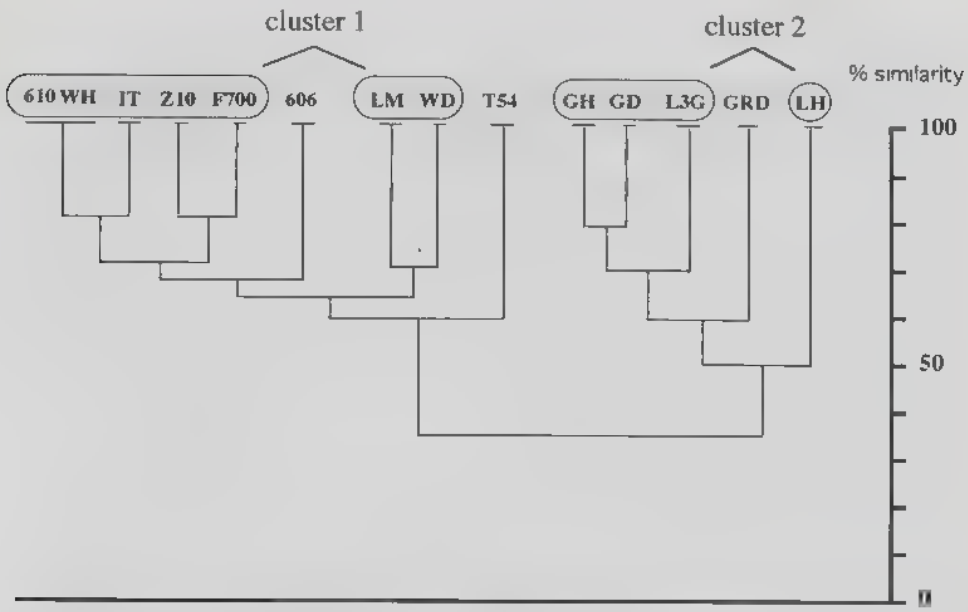

Fig. 7. Dendrogram of Jaccard's similarities for 14 *Stropharia rugoso-annulata* strains based on esterase zymogram

gical variation within this edible wood rotting basidiomycete is not reliable enough to identify commercial varieties (Puschnel, 1969, Zadrazil & Schliemann, 1975, Szudyga, 1978, Delmas, 1989). The present paper describes the first results concerning variability within the commercial mushroom species *Stropharia rugoso annulata*. In this work, the total protein patterns of 14 cultivated strains, selected for their commercial interest, were compared to evaluate intraspecific variability. In addition, esterase enzymes were studied because of the great polymorphism exhibited by the strains for this enzyme system. In view of the lack of information about *Stropharia* proteins, we first had to set out optimal conditions for soluble protein extraction. In this way, it was shown that alkaline Tris buffers were more efficient for mycelial proteins. This observation differs from what was previously described for *Pleurotus* or *Agaricus* basidiocarps (Mouches *et al.*, 1981; Volland Nail, 1981) and shiitake mycelium (Itavaara, 1990). However it is consistent with other works (Royse & May, 1987; May & Royse, 1988).

The diversity among *Stropharia* strains was assessed and, on the basis of the present work, two phenotypic clusters of strains having various origins were identified. The first cluster included seven strains from Belgium, Germany, France and Hungary (IT, 610, Z10, WD, LM, F700, WH), among which the total protein and isozyme patterns revealed only small intraspecific variations. According to total protein patterns, only four strains from Germany and Hungary constituted the second cluster (LH, GH, GD, L3G). However, separation of esterase isozymes showed more variation in this group than in the first one, suggesting that the enzyme analysis is a more valuable tool (as compared to total protein study) for the identification of these strains.

From our classification, 3 strains may be distinguished: the 606 strain because of its low protein content. This particularity was confirmed with a low number of protein

bands. In addition, this strain presents some signs of degeneration (Bonenfant, 1993). The two other strains, T54 and GRD, are characterized by an intermediary position between the two clusters defined from total protein analysis. Even if esterase pattern study suggests they could be related to the strains from the first cluster, we consider that T54 and GRD strains should form specific phenotypes, which represent a large part of the variability in the cultivated species. The cultural potential of these two strains remains to be assessed. The results described in this paper allow us to present a new phenotype of *Stropharia rugoso-annulata* to growers in France and in Belgium where these strains were not used before. As it has been reported earlier (Toyomasu & Zennyozi, 1981, Boisselier-Dubayle, 1983, May & Royse, 1988, Wang & Wang, 1989), our findings confirm protein electrophoresis as a powerful analytical method that provides useful informations for industrial mushroom production: it may be used for evaluation of biological diversity in a species, for breeding work, for the registration of new strains and for quality control. Also, it might allow the detection of mutations in commercially important strains. However, the analysis of total protein patterns appears to be inaccurate and dull, due to the very large number of bands on electrophoretic profiles. For this reason, isozyme analysis seems to be more promising for an accurate identification of mushroom strains, as it has been reported for other species (Boisselier-Dubayle, 1983, Royse & May, 1987, Itavaara, 1988, May & Royse, 1988). In addition, our study of esterase patterns could be complemented with similar analysis carried out on other enzyme systems. For this purpose, a similar analysis of electrophoretic patterns was carried out with malic deshydrogenase (MDH) system. However, MDH isozymes did not allow us to show genetic variations between most of these *Stropharia* strains (Bonenfant, 1993). On the other hand, further work has been carried out in our lab to characterize the fourteen previous strains on the basis of anatomy description and physiology of mycelium development (Bonenfant-Magne *et al*., submitted for publication). Together, both studies provide the first detailed informations on variability within *S. rugoso-annulata* species.

Also, considerably powerful and informative methods such as RAPD or PCR analysis are in use today and have been applied successfully to study intraspecific genetic diversity of organisms (Tingey & Del Tufo, 1993). There is no doubt that such new techniques would allow the description of the genetic diversity in the edible mushroom *Stropharia rugoso-annulata*.

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